

REMARKS

This Preliminary Amendment is filed in response to the Final Action mailed September 16, 2003.

A Request for Continued Examination of the present application is filed herewith.

A Revocation of Power of Attorney With New Power of Attorney and Change of Correspondence Address is filed herewith.

A Petition for an extension of time from the filing of the Notice of Appeal in order to timely file this Preliminary Amendment is filed herewith. To the extent that any additional fees are required to file this Preliminary Amendment the Commissioner is authorized to charge deposit account # 16-1445.

IN THE TITLE

The title of the application has been amended to reflect the subject matter of the claims. The name of the ion channel is consistent with that currently utilized in the art based on an amino acid sequence comparison of SEQ ID NO: 105. See Exhibit A. See also *Schering Corp. v. Amgen Inc.*, 55 USPQ2d 1650, 1654 (Fed. Cir. 2000).

IN THE CLAIMS

Claims 95, 96, and 117 were pending. Claims 1-94 and 97-116 are withdrawn from consideration as directed to non-elected inventions.

Upon entry of this amendment claims 95, 96, and 117 have been amended to better define Applicants' invention; and claims 118-123 are newly added. New claims 118-123 are supported by the specification at page 50, line 25 to page 52, line 26.

No new matter has been added by the claim amendments or newly added claims.

Rejection under 35 U.S.C. § 101

Claims 96, 96, and 117 remain rejected under 35 U.S.C. § 101 because allegedly the claimed invention is drawn to an invention with no apparent or disclosed specific and substantial credible utility. Applicants respectfully traverse this rejection.

It is respectfully submitted that the Office Action has not established a *prima facie* case that the claimed invention is drawn to an invention with no apparent or disclosed specific and substantial credible utility.

To meet the utility requirement the Supreme Court in *Mitchell v. Tilghman*, 86 US 287 (1873), has held a new product or process must be shown to be “operable” – that is, it must be “capable of being used to affect the object proposed”. Cases subsequent to the *Mitchell* decision have not, interpreted this language in *Mitchell* to mean that a patented product must accomplish all objectives stated in the specification. When a properly claimed invention meets at least one stated objective, utility under §101 is clearly shown.” *Raytheon Co. v. Roper Corp.*, 220 USPQ 592,598 (Fed. Cir. 1983), cert. denied, 469 U.S. 835 [225 USPQ 232] (1984). See also *Carl Zeiss Stiftung v. Renishaw*, 20 USPQ2d 1094,1100 (Fed. Cir. 1991). It is respectfully submitted that the teachings of Applicants’ specification and the claims are in full compliance with the utility requirements of §101.

Applicants draw the Examiner’s attention to the teachings at page 9, lines 13-18 which state the biological function of the encoded sequences of the present invention as human ion channels. The identity of SEQ ID NO:105 as a human ion channel receptor provides support for the utility of the present invention. *In re Langer* 831 USPQ 288, 297 (CCPA 1974). makes clear that a §101 rejection may be overcome by suitable proofs that the statement of utility and its scope as defined in the specification are true. The periodical literature has validated that SEQ ID NO: 105 is a 5HT3E receptor, see Exhibit A, as noted above.

It is also noted that in vitro tests also provide a way to overcome a §101 rejection. See *Ex parte Bhide*, 42 USPQ 2D 1441,1448 (USPTO BPA 1996). Such antibody based analytical tests are described at page 52 lines 22-26, page 54, lines 6-11, and page 57, line 29 to page 58, line 10. The described tests and kits may be performed by one of skill in the art. The proofs of utility need only be convincing to one skilled in the art and this is dependent on the facts of each individual case. *In re Buting*, 169 USPQ 689,690 (CCPA 1969).

In addition, The United States Patent Office has long recognized the utility of antibodies directed to specific amino acid sequences, see for example, U.S. Patent Nos. 6,545,129 B1; 6,503,733 B1; 6,277,377 B1; and 6,228,616 B1. These patents

were noted by performing a key word search of U.S. issued patents having claims including the terms: specific; antibody; sequence; and human. The search results yielded greater than 300 hits.

The Office Action relies on the decision of *Brenner v. Mason* to support the instant §101 rejection of the claims for lacking utility. Applicants respectfully disagrees with this position as the *Brenner* decision was directed to a set of distinct facts and issues developed in the patent application under appeal. The Supreme Court in *Brenner* in construing the term “useful” in §101 of the Patent Act noted that:

“As is so often the case, however, a simple, everyday word can be pregnant with ambiguity when applied to the facts of life. That this is so demonstrated by the present conflict between the Patent Office and the CCPA over how the test is to be applied to a chemical process which yields an already known product whose utility – other than as a possible object of scientific inquiry – has not yet been evidenced.”¹

In view of the foregoing, Applicants respectfully requests reconsideration of the Office Action mailed September 16, 2003.

Rejection under 35 USC §112 first paragraph

Claims 95, 96 and 117 were rejected under 35 USC §112 first paragraph on the basis that the claimed invention was not supported by either a clear asserted utility or a well-established utility for reasons set forth in the rejection of the claims under 35 U.S.C. §101 for lack of utility. Applicants respectfully traverse this rejection.

It is respectfully submitted that the Office Action has not established a *prima facie* case that the claimed invention is not supported by either a clear or asserted utility or a well-established utility that one skilled in the art would not know how to use the claimed invention.

Applicants note that as the requirements for utility under §101 have now been properly met for the reasons stated above, the instant rejection under 35 U.S.C. §112, first paragraph should thus be mooted.

¹ *Brenner v. Mason*, 148 USPQ 689,693 (US 1966).

Applicants teaching of SEQ ID NO:105 provides to the public how the claimed invention can be utilized. See *Noelle v. Lederman*, 62 USPQ2d. 1508, 1514 (Fed. Cir. 2004).

In view of the foregoing Applicants respectfully request reconsideration of the Office Action mailed September 16, 2003.

Rejection under 35 U.S.C. § 103

Claims 96, 96, and 117 remain rejected under 35 U.S.C. § 103(a) as allegedly unpatentable over Isenberg *et al.* (Neuroreport, 1993 5:121-124). The Office alleges that “because the amino acid sequence of 5HT3 receptor disclosed by Isenberg et al. comprises an epitope of eight consequent amino acids, which completely match an epitope of SEQ ID NO: 105, an antibody generated against the sequence, which comprises this epitope, would also bind the polypeptide of SEQ ID NO: 105 of the instant invention. One of ordinary skill in the art would be motivated to generate antibodies against 5HT3 receptor using the entire receptor sequence, which would include the epitope identical to SEQ ID NO: 105.” (Office Action, page 6). Applicants respectfully disagree.

It is submitted that the Office Action has failed to demonstrate a *prima facie* case of obviousness for the pending claims. See MPEP §2143.

As the Office previously acknowledged, Isenberg does not disclose an antibody that binds to a fragment of the 5HT3 (now recited as 5HT3E) receptor (Office Action, mailed 1/29/03). Applicants respectfully point out that even if Isenberg did disclose an antibody that binds to the receptor, it would have to be demonstrated that the antibody would also bind to an epitope within SEQ ID NO: 105 for it to make obvious the claimed invention. The Office Action has failed to identify, in this or any Office Action, an antibody that can bind to the epitope that the Office Action has identified. The polypeptide discussed in Isenberg has many epitopes and even if one of skill in the art were motivated to generate antibodies against that polypeptide, it is not clear from the rejection why one of skill in the art would choose the recited 8 amino acids to generate the antibody, nor would there be no expectation of success by one of skill in the art that an antibody would be generated that binds to the 8 amino acid sequence and would also bind to the then unknown sequence in SEQ

ID NO: 105. Without the expectation of success the present claims cannot be obvious to one of skill in the art.

It is submitted that the Applicants herein first isolated SEQ ID NO: 105. The Isenberg reference fails to teach or even suggest SEQ ID NO:105 or generating antibodies that are “specific for” SEQ ID NO:105. Accordingly, the pending claims are not obvious in view of the Isenberg reference.

In view of the foregoing, Applicants request that the rejection under 35 U.S.C. § 103(a) be withdrawn.

Rejection under 35 USC §112 second paragraph

Claim 117 was rejected under 35 U. S. C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. Applicants respectfully traverse this rejection.

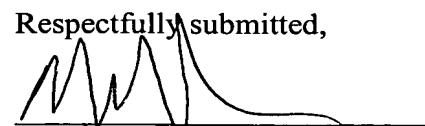
Claim 117 has been amended to moot this rejection.

In view of the foregoing, Applicants request that the rejection under 35 U.S.C. § 112, second paragraph, be withdrawn.

Conclusion

Applicants believe the claims are in condition for allowance. An early Notice of Allowance is therefore earnestly solicited. Applicants invite the Examiner to contact the undersigned to clarify any unresolved issues raised by this response.

Respectfully submitted,



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Cloning, physical mapping and expression analysis of the human 5-HT₃ serotonin receptor-like genes *HTR3C*, *HTR3D* and *HTR3E*[☆]

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Received by M. D'Urso

Abstract

For more than 50 years the serotonin system has been the subject of intense research. This has provided an exciting insight and led to the discovery of multiple drugs targeting serotonin receptors, metabolising enzymes and re-uptake sites. During the past few years researchers focussed especially on elucidating the complexity of different physiological actions in the serotonergic network. We have identified two novel human serotonin 5-hydroxytryptamine type 3 receptor-like genes, *HTR3D* and *HTR3E*, by performing homology searches using the public human sequence databases and subsequently cloned the full length cDNAs by 5' and 3' rapid amplification of complementary DNA ends. Mapping of *HTR3D* and *HTR3E* by hybridisation, polymerase chain reaction and fluorescence *in situ* hybridisation revealed that both genes together with *HTR3C* are clustered in a subinterval of less than 100 kb on chromosome 3q27. Comparative expression analysis of all *HTR3* genes, namely *HTR3A*, *B*, *C*, *D* and *E* showed *HTR3D* expression to be restricted to kidney, colon and liver and *HTR3E* expression to colon and intestine, whereas all other genes are widely expressed in many tissues including brain.

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Keywords: Serotonin receptor genes; 5-Hydroxytryptamine; Ligand-gated ion channel; *HTR3C*; *HTR3D*; *HTR3E*; Chromosome 3q27

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) controls a variety of physiological functions in the central and peripheral nervous systems. The serotonergic system is organised in a highly complex manner, as serotonin action is mediated by a multitude of 5-HT receptor subtypes. These subtypes can be divided into seven main classes (5-HT_{1R}–5-HT_{7R}) based

on their structural and functional features (Hoyer et al., 2002). This high degree of receptor variability clearly emphasises the physiological importance of serotonin and points to an extraordinary diversity of functions. The unravelling of the properties of the system leading to this complexity is one of the major goals of serotonin research.

Except for the 5-HT₃ receptor, which is a ligand-gated ion channel, all serotonin receptors represent G-protein coupled binding proteins. However, the 5-HT₃ receptor shares characteristic features with the other members of this ion channel family: a large extracellular domain containing a conserved cysteine loop, four hydrophobic transmembrane segments, a large intracellular loop between the third and fourth transmembrane region and an extracellular C-terminus (Reeves and Lummis, 2002). The ion channel itself is an oligomeric complex composed of five subunits. During the last few years two different human 5-HT₃ receptor subunit genes, *HTR3A* and *HTR3B*, have been isolated (Miyake et al., 1995; Davies et al., 1999).

Several studies pointed out the complexity within the 5-HT₃ receptor system based on indications of pharmacological and biophysical variations between tissues and

* After submission of our paper similar work has been presented at the Neuroscience Meeting; Gotow et al. (2002): a cluster of novel 5-HT₃ receptor-like genes on chromosome 3; Poster 38.1.

Abbreviations: 5-HT, 5-hydroxytryptamine; 5-HT₃, receptor, 5-hydroxytryptamine receptor type 3; cDNA, complementary DNA; BAC, bacterial artificial chromosome; EST, expressed sequence tag; FISH, fluorescence *in situ* hybridisation; FITC, fluorescein isothiocyanate; HEK293, human embryonic kidney 293; *HTR3*, 5-hydroxytryptamine receptor type 3 gene; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction.

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species. Different ligand binding affinities and ion channel conductances of diverse tissues and cell lines have been reported and pharmacologic profiles of homomeric and heteromeric receptors showed differences in the properties of the respective receptors. Only 5-HT_{3A} subunits can form functional homo-oligomeric complexes after expression in *Xenopus* oocytes or HEK293 cells, whereas the 5-HT_{3B} subunits are not able to build a functional receptor on their own (Miyake et al., 1995; Davies et al., 1999; Dubin et al., 1999). Recombinant homo-oligomeric 5-HT_{3A} receptors show extremely reduced channel conductances compared to native receptors, whereas heteromeric complexes composed of both 5-HT_{3A} and 5-HT_{3B} subunits resemble closely the properties of the native receptors (Davies et al., 1999). Variable receptor compositions have also been shown by Western blot experiments which led to the hypothesis that further 5-HT₃ receptor subunits actually should exist (McKernan, 1992; Hussy et al., 1994; Jackson and Yakel, 1995; Fletcher and Barnes, 1998). To verify this hypothesis we have established a 5-HT₃ receptor consensus sequence to screen databases and identified a human BAC clone (RP11-315J22) harbouring additional subunit sequences. We established primers and cloned the entire coding region of two novel *HTR3*-homologous genes by 5' and 3' RACE: *HTR3D* and *HTR3E*. Mapping data of *HTR3D* and *HTR3E* and of the recently cloned *HTR3C* gene (accession number AF459285) revealed that they all reside in close vicinity on chromosome 3q27. To get a better insight into the putative role of *HTR3D* and *HTR3E*, we carried out expression analysis and compared the expression profiles to those of *HTR3A*, *B* and *C*.

2. Material and methods

2.1. Establishment of the 5-HT₃ receptor consensus sequence

Multiple sequence alignment with the protein sequences of the human 5-HT_{3A} and 5-HT_{3B} subunits, as well as the 5-HT_{3A} sequences of rat, mouse and guinea pig (Uetz et al., 1994; Isenberg et al., 1993; Miyake et al., 1995; Lankiewicz et al., 1998; Davies et al., 1999) was carried out by the MALIGN algorithm of the Biocomputing home page (<http://genius.embnet.dkfz-heidelberg.de/>). Using this alignment we created a 5-HT₃ consensus sequence by the Profilemake algorithm. This 5-HT₃ consensus sequence (Fig. 1A) was subsequently used for searching the human genome database (<http://www.ncbi.nlm.nih.gov/BLAST/>), the Celera database (<http://publication.celera.com/cds/login.cfm>) as well as EST databases by the tblastn algorithm (<http://www.ncbi.nlm.nih.gov/BLAST/>: protein query – Translated db [tblastn]).

2.2. PCR analysis

PCRs were performed in 50 µl volumes containing 10–100 ng template, 25 pmole of each primer, 200 µM dNTPs (MBI Fermentas), 1.5 mM MgCl₂, 1 × PCR buffer and 2 U HotStarTaq DNA Polymerase (Qiagen). Thermal cycling was carried out in a Thermocycler PTC-200 (MJ Research) under the following conditions: initial denaturation at 94°C for 15 min followed by 35–40 cycles of 94°C for 30 s, annealing temperature (T_A) for 30 s and 72°C for 2 min. Final extension was carried out at 72°C for 5 min. Primer sequences and annealing temperatures are given in Table 1.

2.3. Rapid amplification of cDNA ends (5' and 3' RACE)

To clone the 5' and 3' ends of *HTR3D* and *HTR3E*, 5' RACE was performed using 'Marathon cDNA libraries' constructed as described by the manufacturer (BD Bioscience Clontech). The following oligonucleotide primers (Table 1) were used: 5'RACE: HTR3D2rev/HTR3E1rev; 3'RACE: HTR3D4for/HTR3D5for and the adaptor primer AP1. PCR was carried out using the following parameters: 94°C for 15 min, followed by 25 cycles of 94°C for 30 s, 55/60°C for 30 s, 72° for 2 min, final extension at 72°C for 5 min. A second round of PCR was performed using 1/50 of first PCR product and the following nested oligonucleotide primers: 5'RACE: HTR3D2ev2/HTR3E1rev2; 3'RACE: HTR3D4for2/HTR3D5for2 and the adaptor primer AP2. PCR was carried out at an annealing temperature of 55°C for 35 cycles.

2.4. Sequencing

2.4.1. Cloning and sequencing of PCR products

PCR products from cDNAs showing positive hybridisation signals were cloned into the pCRII Topo vector (pTOPO kit, Invitrogen). Overnight cultures of single colonies were lysed in 100 µl H₂O by boiling for 10 min. The lysates were used as templates for PCRs with the respective vector primers. Both strands were sequenced with the Cy5-labelled vector primers *M13 universal* and *M13 reverse* according to manufacturer's protocols (Cycle Reader Kit, MBI Fermentas) on an ALFExpress automated sequencer (Pharmacia) or using unlabelled receptor sequence specific primers (Table 1, sequencing primers indicated with the extension 'S' in the primer name) on a MEGABACE Sequencer (Pharmacia).

2.4.2. Sequencing of BAC clones

In order to identify missing exon-intron junctions which were not available in the public databases, we carried out partial sequencing of the BAC clones RP11-810O14 and CTD-2545A22 using Cy5-labelled primers (Table 1;

A

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1  MMLWIPQVLL ALFLPTILLAQ GEGRRRRATQ AHNTTQPALL RLSBHLLANY
51   RKGVRPVRBW RKPTTVSIBV IVYAILNVBE KNQVLTTYIW YRQYWTREFL
101  QNNPEDEFNI TKLSEIPTDSI WVEBILINEF VBVGKSPNIP YVYVHHGGEV
151  QNYKPLQVVVT ACSLBIIYNFP FBVQNCSTTF TSWLNTIQBI NISLWRSPPE
201  VKSBKSVFVNQ GEWELLGVF PQFKEFSBIE SSNSYAEMKF YVVIIRRPLF
251  YAVSLILPSI FLMVVBIVCF ZIFPESSEERV SFRITLLNSY SVFLITVSRT
301  LPATAIGTPL EGVYFVVCMQ LIVICLAETI FIVRLVHKQS DQQPVFRWLR
351  HLVLRLRIAWL LCLSEDOFTAH RPPATFOANK TSECGCGELL RAMGNBTSHV
401  GGPQBLEKTP RGRGGPPFCPP REASLAVCGL EQELASSIRNF LEKRSEMREV
451  ARKWLAVGGSV LSELLFRIVL LAVLAYSITL VTLWSIWHYA

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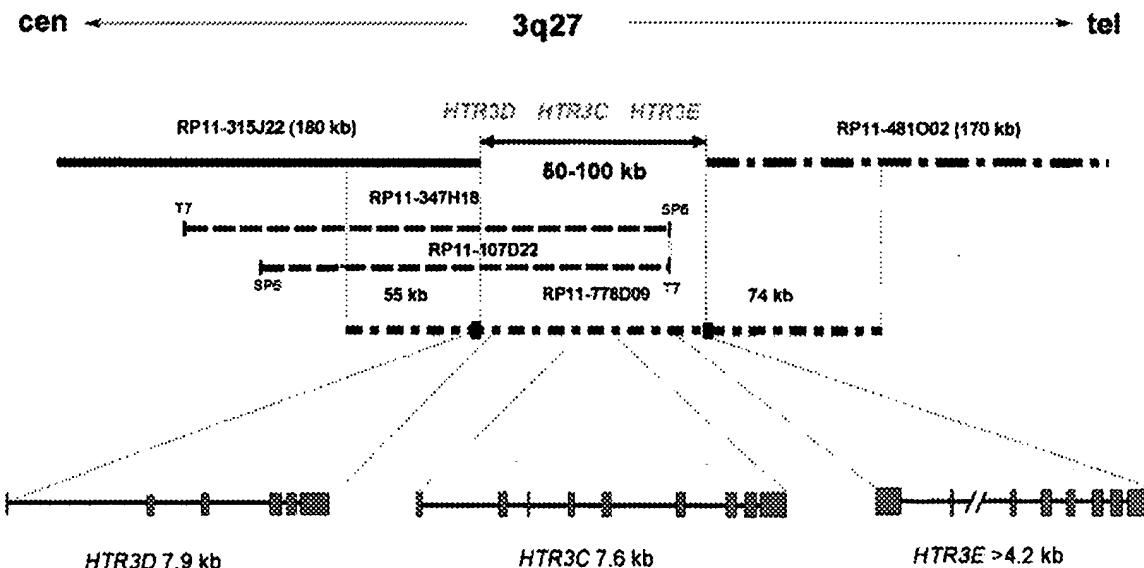
B

Fig. 1. (A) A 5-HT₃ receptor consensus sequence was created based on the amino acid sequence of the human 5-HT_{3A} and 5-HT_{3B} subunits, as well as the 5-HT_{3A} protein sequences of rat, mouse and guinea pig. (B) Genomic organisation and localisation of the *HTR3* genes *HTR3C*, *HTR3D* and *HTR3E* on chromosome 3q27. BAC clones RP11-347H18, RP11-107D22, RP11-778D09 (AC131235) and RP11-481O02 (AC04831) are not completely sequenced, which is indicated by dotted lines. RP11-347H18, RP11-107D22 are in the database as genomic survey sequences (GSS: AQ542473, AQ542471; AQ315444; AQ315441). RP11-315J22 (AC068644) is completely sequenced. The size of intron 2 in *HTR3E* is unknown.

Table 1
HTRC, *HTR3D* and *HTR3E* specific primers

HTR3C	HTR3D	HTR3E	Primer name	Sequence (5' → 3')	Primer name	Sequence (5' → 3')
e1	-	-	HTR3C5'UTRfor	TCC CCA GAG AAC AGT CCA GA	HTR3CE1rev2	CCC TCC AAG TGC TAG AGG TG
	CDS	-	HTR3C5'UTRfor	TCC CCA GAG AAC AGT CCA GA	HTR3C7UTRrev	GAC CAG CAG AAA CTC CAA GC
	-	-	HTR3Diex1RACE	GGC TAG ATT CAG GCC CAG TTA AAG	HTR3D5rev2	CTG CCT AGG TGT TCC AGA GG
	-	-	HTR3D1for	CTG CAG CCT TTC AAG CAG T	D5rev	CTG CCT AGG TGT TCC AGA GGC AT
	-	-	HTR3D2rev	GGG AAA TAG AAG GPG TCC AGT TTG	API	CCA TCC TAA TAC GAC TCA CTA TAG GGC
	-	-	HTR3D2rev2	GTG TCC AGT TTG CAG ATG CTG AC	AP2	ACT CAC TAT AGG GCT CGA GGC GC
	-	-	HTR3D5for	AGC TGT GGG TGC AGT TCA G	API	CCA TCC TAA TAC GAC TCA CTA TAG GGC
	-	-	HTR3D5for2	CTC CAT CAC CGT CAT ATG	AP2	ACT CAC TAT AGG GCT CGA GGC GC
	-	-	HTR3D1for	CTG CAG CCT TTC AAG CAG T	HTR3D1rev	ATG GCA GAC GTG AAG GA
	-	-	HTR3D2for	ATA AGC CAA TGT GGG TGC TC	HTR3D2rev	GGG AAA TAG AAG GTG TCC ACT TTG
	-	-	HTR3D5for	TGG TAA ACT TTC TGG TGC CC	HTR3D5rev	TGG GAG CAA GTC ATT CAT CA
	-	-	HTR3D4for	GTG TCT ACT TCG CCC TGT GC	HTR3D4rev	GCG CCT TAT TTC CCT TCT GG
	-	-	HTR3D5for	AGC TGT GGG TGC AGT TCA G	HTR3D5rev	CTG CCT AGG TGT TCC AGA GGC AT
	-	-	HTR3D4for	GTG TCT ACT TCG CCC TGT GC	API	CCA TCC TAA TAC GAC TCA CTA TAG GGC
	-	-	HTR3D4for2	TGC CTG CTC CTC ARG GTC GG	AP2	ACT CAC TAT AGG GCT CGA GGC GC
	-	-	CDS	ATG TGA GCT TGT ACT TPA CGG GC	HTR3D5rev	CTG CCT AGG TGT TCC AGA GGC AT
	-	-	3'UTR	GCT TCT CTT GCC TCC AGG G	3'UTR_HTR3Erev(S)	AAG AGG TAT AGT CTC CTA TGC
	-	-	3'UTR	TAC CTG ACA CAC AGC CAG TGC	HTR3E1inREV(S)	TAAG GAA CAC CAA GGG CTA
	-	-	3'UTR	GCG AAG GAG ATG TPG ACT TTG G	API	CCA TCC TAA TAC GAC TCA CTA TAG GGC
	-	-	CDS	GTT GGT GAC CGG AGC GAA GG	API	CCA TCC TAA TAC GAC TCA CTA TAG GGC
	-	-	3'UTR	CAA TGA TGA AAA TGT CTG GG	HTR3EE2forS	GGA ACC CAG AGG AAT GTG AG
	-	-	3'UTR	TGA GAG AAT AAA GTG TPG TCC A	-	-
	-	-	3'UTR	TGT TTC AAA CCA AAG TPA GGG A	-	-
	-	-	3'UTR	GGA TGG AAA AAG AGT GCA GT	HTR3EE3inREV	CTT GGG AGA TAC ATA TTT GAT G
	-	-	3'UTR	GGT TCC TCT GAC CCC ATA ACT	HTR3E5inREV(S)	CCC ACC CTT CTC TTC CAA AA
	-	-	3'UTR	CCT CTG GCC CTC ACT AGG C	HTR3D4rev	GGC CCT TAT TTC CCT TCT GG
	-	-	3'UTR	HTR3E6inFOR	-	-

Sequencing primers are indicated by an (S) and were labeled by Cy5. As *HTR3C*, *D* and *E* are highly homologous within their 3' part, *HTR3D* specific primers could be used for RACE, RT-PCR and PCR experiments amplifying *HTR3D* and *HTR3E*, respectively. CDS: coding sequence; e: exon; i: intron; hcDNA: hypothetical predicted *HTR3D* cDNA; UTR: untranslated region; T_A: annealing temperature.

sequencing primers indicated with the extension ‘S’ in the primer name) according to manufacturer’s protocols (Cycle Reader Kit, MBI Fermentas) on an ALFExpress automated sequencer (Pharmacia).

2.5. Expression analysis

Fifty ng Poly(A)⁺ RNAs from 18 different tissues (adult: brain, amygdala, caudate nucleus, hippocampus, thalamus, colon, intestine, kidney, liver, lung, heart, muscle, spleen, stomach; fetal: brain, colon, kidney, muscle) were reverse transcribed using the superscript system from Gibco BRL as described by the manufacturer. PCR analysis was performed using different gene specific primers (Table 1) as described above.

2.6. Genomic clones and physical mapping

BACs were derived from the Resource Center, Oakland, USA (RPCI-11, Roswell Park Cancer Institute Human BAC Library) or from Research Genetics, Invitrogen Corporation, Breda, The Netherlands (CTD). Bacteria bearing the respective BAC clones were grown overnight at 37°C in LB medium (25 µg/ml chloramphenicol). BAC DNA was isolated using the Nucleobond Maxi Preparation Kit from Macherey and Nagel as recommended by the manufacturer. Clones were digested with 200 U *Eco* RI in the appropriate buffer in a 100 µl volume. Digested BACs were run on a 0.8% agarose gel. Overlapping clones were confirmed by PCR and hybridisation analysis.

2.7. Southern blot hybridisation

Southern blot hybridisations were carried out using respective probes containing the conserved regions of *HTR3C*, *D* and *E*: an exon 1 probe of *HTR3C*, 3’UTRs of *HTR3D* and *E* in hybridisation buffer (5 × SSPE, 10 × Denhardt’s, 2% SDS, 20 ng/ml herring sperm DNA) at 65°C overnight and washed twice in 2 × SSC, 0.5% SDS and once in 0.2 × SSC, 0.1% SDS at 65°C. Filters were exposed to Kodak X-omat films at –80°C or room temperature for several minutes/hours.

2.8. Fluorescence *in situ* Hybridisation (FISH) analysis

Biotinylated BAC DNAs of the clones RP11-315J22, RP11-778D09, RP11-810O14, RP11-107D22, RP11-347H18 and CTD-2545A22 were hybridised to metaphase chromosomes from control individuals under conditions as described before (Lichter and Cremer, 1992). To confirm the localisation of the BAC clones on chromosome 3, a control BAC probe (CIT-B 159N23) partially harbouring the MEGAP gene residing on chromosome 3p25 was used (Endris et al., 2002). The hybridised probes were detected via avidin-conjugated FITC and anti-digoxigenin-conjugated Cy3.

2.9. Bioinformatics

Multiple sequence alignments were performed using MALIGN and CLUSTAL from the HUSAR program package on the Biocomputing home page (<http://genius.embnet.dkfz-heidelberg.de/>). Sequence comparisons and homology searches were carried out using GAP and BESTFIT from the HUSAR program package and different BLAST algorithms at the NCBI web site (<http://www.ncbi.nlm.nih.gov/BLAST/>). Phylogenetic trees were created using the evolutionary analysis software at the Biocomputing home page. Sequence analysis of genomic sequences was carried out with the NIX analysis software (<http://www.hgmp.mrc.ac.uk/Registered/Webapp/nix/>).

Pattern and profile searches as well as hydrophathy analysis for the transmembrane topology were done by Prosite and TMHMM at the Biocomputing home page. Signal peptide cleavage sites and theoretical peptide mass were predicted by the Peptide characterisation software on the ExPasy server (<http://www.expasy.ch>) and by using the Sigfind Software (Signal Peptide Prediction Server: <http://www.stepc.gr/~synaptic/sigfind.html>).

3. Results

3.1. Identification of novel 5-HT₃-like receptor subunit genes

To investigate the existence of further 5-HT₃ receptor encoding genes in the human genome, we established a consensus sequence (Fig. 1A) taking advantage of amino acid sequences of the human 5-HT_{3A} and 5-HT_{3B} subunits, as well as the 5-HT_{3A} protein sequences of rat, mouse and guinea pig to search the public databases for homologous sequences (Uetz et al., 1994; Isenberg et al., 1993; Miyake et al., 1995; Lankiewicz et al., 1998; Davies et al., 1999). Using this consensus sequence, we were able to identify the human BAC clone RP11-315J22 located on chromosome 3q27 that enclosed short intervals encoding homologous protein stretches between 35 and 99 amino acids in length and similarities of 45–72%. Further sequence analysis of the RP11-315J22 genomic sequence by FGENES, Genescan and Genefinder with the NIX analysis software (<http://www.hgmp.mrc.ac.uk/Registered/Webapp/nix/>) predicted several exons of an *HTR3* homologous gene of excellent quality.

3.2. Cloning of full length cDNAs

Based on the genomic sequence we designed primers residing in the most upstream and downstream putative exons (*HTR3D*1for/*HTR3D*5rev). By RT-PCR we were able to amplify a 1.8 kb product from kidney. Using 5' and 3' RACE succeeded in cloning the entire coding region of not only one but two novel cDNAs. We named the new homologous sequences *HTR3D* and *HTR3E* (AY159812;

AY159813), since a gene termed *HTR3C* has recently become available in the database (accession number AF459285). The cDNA of *HTR3D* spans 1499 base pairs; the coding region of 840 bp (position 227–1066) encodes a predicted protein of 279 amino acids. The complete cDNA of *HTR3E* is 1925 base pairs in length; the coding region spans 1416 bp (position 195–1610) and encodes a predicted protein of 471 amino acids. In comparison, the complete cDNA of *HTR3C* is 1745 base pairs in length; its coding region spans 1344 bp (position 35–1378 of AF459285) and encodes a predicted protein of 447 amino acids.

To investigate the evolutionary relationship between *HTR3C*, *D* and *E*, we carried out sequence comparison on nucleotide and amino acid level. Comparison on the nucleotide level revealed overall homologies of 82.8–90.1% between the three cDNAs. Comparison of *HTR3C*, *D* and *E* to the previously known genes of the *HTR3* class, *HTR3A* and *HTR3B*, showed that similarity is much lower (Table 3). On protein level the 5-HT_{3C}, 5-HT_{3D} and 5-HT_{3E} subunits show identities between 64.8 and 74.3%, while their identity compared to 5-HT_{3A} and 5-HT_{3B} ranges from 26.8 to 39.8% (Table 3). It is very unlikely that the novel genes represent members of the nicotinic acetylcholine receptor (nAChR) family. The nAChR subunits most closely related are alpha 9 and alpha 10 revealing overall identities of only 27%. We have established dendograms based on multiple sequence alignments using all known *HTR3* cDNAs of human and other species. These data

strongly suggest that *HTR3C*, *D* and *E* share a common evolutionary history, whereas *HTR3A* and *HTR3B* are evolutionary more distant.

3.3. Analysis of the encoded protein sequences

Analysis of the 5-HT_{3C}, 5-HT_{3D} and 5-HT_{3E} subunits revealed a signal peptide sequence for 5-HT_{3C} and 5-HT_{3E} encompassing 27 and 16 amino acids. Both subunits contain a cysteine loop at the extracellular N-terminal part of the protein. In contrast, 5-HT_{3D} shows no signal peptide sequence and a very short extracellular N-terminus lacking the cysteine loop. All three subunits contain four transmembrane regions. The C-terminus of the protein is predicted to reside extracellular in the case of 5-HT_{3C} and 5-HT_{3E} and it would be located intracellular for the 5-HT_{3D} subunit. The analysis of the protein sequences using the Prosite program predicted several putative Asn-glycosylation, PKC, CKII, cAMP and tyrosine kinase phosphorylation sites (Fig. 4). The theoretical mass of the encoded subunits was calculated using the Peptide Mass Peptide Characterisation Software on the ExPasy server as follows: 5-HT_{3C}: 48 kDa, 5-HT_{3D}: 30 kDa and 5-HT_{3E}: 52 kDa.

3.4. Physical mapping of *HTR3C*, *HTR3D* and *HTR3E*

In addition to searching the HUMANace-Human Genomic Physical Map Tracking Database (<http://genome.wustl.edu>)



Fig. 2. Fluorescence *in situ* hybridisation on metaphase chromosomes of a human male using the BAC clone RP11-778D09 harbouring the *HTR3* genes *HTR3C*, *HTR3D* and *HTR3E*. The clone displayed signals on the long arm of chromosome 3 band 3q27. The localisation on chromosome 3 was verified by co-hybridisation with the BAC probe CIT-B 159N23, that partially encompasses the MEGAP gene on 3p25 (Endris et al., 2002).

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edu/cgi-bin/ace), BLASTN analysis using sequence data of RP11-315J22 and the *HTR3D/E* cDNA sequences led to the identification of distally overlapping BAC clones (RP11-778D09, RP11-810O14, RP11-107D22, RP11-347H18, CTD-2545A22) which had all been pre-assigned to chromosome 3q27 (four of them are indicated in Fig. 1). FISH analysis of all six clones confirmed their chromosomal localisation to 3q27. Fig. 2 depicts the localisation of clone 778D09. Physical mapping of *HTR3C*, *HTR3D* and *HTR3E* by hybridisation and PCR analysis showed that all three genes map within an interval of less than 100 kb in the following order proximal to distal: *HTR3D*, *HTR3C*, *HTR3E* (Fig. 1).

3.5. Genomic organisation of *HTR3C*, *D* and *E*

Exon-intron organisation of all three *HTR3* genes was determined using sequence data available in the human genome and the Celera databases and the SIM4 algorithm (<http://pbil.univ-lyon1.fr/sim4.html>) (Table 2). In cases where exon-intron boundary sequences were missing in the database, we carried out partial genomic sequencing using the respective BAC clones. All of the determined exon-intron junctions are consistent with the consensus splice site sequences (Breathnach and Chambon, 1981).

A comparison of the cDNAs with the genomic sequence

reveals that *HTR3C* has nine exons (45–522 bp), whereas *HTR3D* is composed of only six exons (62–557 bp). *HTR3E* contains eight exons (45–527 bp) (Table 2, Fig. 1). In all three cases the last three exons of the respective genes are separated by only very small introns of less than 200 bp (Table 2). Exon sizes as well as exon-intron junctions of *HTR3C* and *E* are almost identical. Both also contain a similar number of exons (9 and 8). In contrast, *HTR3D* shows a completely different genomic organisation with only six exons. All three genes are small, between at least 4 kb for *HTR3E* and about 8 kb in the cases of *HTR3C* and *HTR3D* (see Fig. 1).

3.6. Expression analysis

We performed comparative expression analysis by RT-PCR amplifying the complete coding regions of all *HTR3* genes using a variety of cDNAs prepared from human adult and fetal tissues (Fig. 3). Hybridisation probes specific for each *HTR3* gene were used to confirm the PCR products. Expression of *HTR3D* and *HTR3E* was shown to be confined to only three and two tissues, respectively (*HTR3D*: adult: kidney, colon, liver; fetal: colon and kidney; *HTR3E*: adult: colon, intestine). *HTR3C* was expressed in many more tissues (adult: brain, colon, intestine, lung, muscle, stomach; fetal: colon and kidney). Therefore, the expression

Table 2
Exon-intron boundaries of *HTR3C*, *HTR3D* and *HTR3E*

Exon	Intron	Exon	Intron	Exon size (bp)	Intron	Intron size (bp)
<i>HTR3C</i>						
1	...gggtttgggagctcc	TGGTGAATCC...CTGCTTCAG	gtaaaggatgggacgagaacag...	101	1	1575
2	...ggagtctctgtctctatag	GAAGAGGGGA...CCTGGGAGTG	gttgagacttgttgcctcat...	167	2	424
3	...agtggaaatttccttgcag	GATGCACAGC...GATGGATTG	gttaaggcagatcaactatct	45	3	820
4	...ccctcaactgcccgtatcg	GTATGGAC...TCGGAAATC	gtgcgtatgcaggctggga...	110	4	588
5	...tcctcccttccccaaacag	CATGGATGTG...CTCTACACAG	gttaagtgtacacattttgg...	170	5	1382
6	...ctgaccggcctcccttcccg	TGCGACAGCAT...CATGTTTAT	gttgagttccggccctgt...	161	6	848
7	...cttctccggctctcccttcccg	GTGGCCATCA...CCCCTCATCA	gtatggctctccacttct...	205	7	187
8	...ataatttgcgttgccttcag	GTGCTACTT...CACCTGCCGT	gtgaggaaagccacatgt...	216	8	106
9	...agctctgttcttcacacag	GCCCCAAAGGA...ACTMTCCAGG	aagcaactggcttc...	522		
<i>HTR3D</i>						
1	...cacatattttgttctct	TCTCCAAACT...AAAGCACAGG	gttgagttatctctgtgac...	28	1	3485
2	...tgatctacatcattttcag	TGGCTTAGAT...GCTAAATATG	gtatgacagacttgtttcc...	167	2	1146
3	...cctgtcttctccacacag	CATCACTGTG...ACCTACACAG	gttaagtgggtcaactaaag...	172	3	1480
4	...ctccttcttctcccccacag	GTGGCCATCA...CAAAGCAG	gtgtgttgtggatgggaga...	253	4	135
5	...gcagaccccttgccttcag	GTGCTACTT...CACCTGCCCG	gtgaggaaagtcatacttcc...	216	5	121
6	...gagtctctgttcttcgttag	GTGTGAAGGA...GATTTCTCTT	acgtgtgttttttaagt...	635		
<i>HTR3E</i>						
1	gcagacaaaacctgggtca	GAACAAGTCC...CCTAGATGTG	gttgatgtgtggccatcttag...	473	1	833
2	...atagggaaattttttggcag	AATGAAACAGC...GCTGGAAATG	gtatggacaaacactttatcc...	45	2	?
3	...ctcctccacctgggtcttag	GTTTGGGATA...TCATTTGACT	gttcgtatcaagggtgtgtc...	110	3	495
4	...ttgcgttatctctcccttcag	CATGGATGTG...CTCTACACAG	gttaagtgtcgatgtgggttc...	170	4	310
5	...cagatgggttcattttcag	TGGACAGCAT...CGTGTCTAT	gtgagttggggatcttac...	161	5	337
6	...acccttccttacccacacag	GTGGCCATCA...CCCCTCATCG	gtatggctctccacatttt...	205	6	158
7	...taggcccccttcccttcag	GTGCTACTT...CACCTGCCCG	gtgaggaaagtcatacttct...	216	7	120
8	...gagtctctgttcttcgttag	GTGTGAAGGA...AATTCCTGCAG	agatttctggctttttgtca...	527		

Exon sequences are shown in capital letters, donor and acceptor splice sites are indicated in bold type. The size of intron 2 in *HTR3E* is unknown.

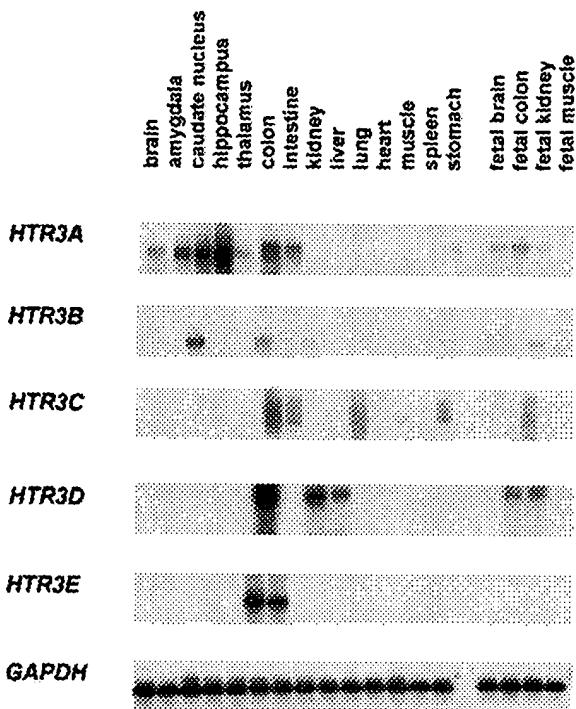


Fig. 3. Expression analysis. RT-PCR analysis of the novel genes *HTR3D* and *HTR3E* compared to *HTR3A*, *HTR3B* and *HTR3C* using cDNAs from 18 different human fetal and adult tissues. Sizes of PCR products were 1.5 kb for *HTR3A*, 1.4 kb for *HTR3B*, *HTR3C* and *HTR3E* and 1.1 kb for *HTR3D*. *GAPDH* expression was analysed as a control for cDNA integrity.

pattern of *HTR3C* resembles more closely the expression profiles of *HTR3A* and *HTR3B* (Miyake et al., 1995; Davies et al., 1999).

4. Discussion

Neurons within the nervous system are organised in different neural networks through synaptic connections. Two fundamental components interact dynamically in these functional units: the neurons themselves and their synapses. Identifying cellular and synaptic properties is necessary to elucidate the links between neural network behaviour and physiological function and represents a useful step towards a better understanding of neurological diseases. Especially the serotonergic system has been in the centre of interest as it is implicated in a multitude of signalling processes and in the etiology of several diseases. One of the main goals of serotonin research is to enlighten the complexity of the system, in particular of receptor diversity and its underlying mechanisms. Complexity can be achieved on different levels: on the genomic level through different subtypes, on the expression level through different splice variants and on the protein level through post-translational modifications. Additionally, oligomerisation increases the possible

composition of interacting molecules in the network as shown in different ligand-gated ion channels such as acetylcholine, GABA and 5-HT₃ receptors (Riordan, 1992; Colquhoun and Patrick, 1997; Morales et al., 2001; Pootanakit and Brunken, 2001; Morales and Wang, 2002).

We have investigated whether further 5-HT₃ receptor genes exist in the human genome. Two novel *HTR3* homologous genes, which we termed *HTR3D* and *HTR3E* (since *HTR3C* has been recently accessible through the database) were isolated. Analysis of the putatively encoded 5-HT_{3C}, 5-HT_{3D} and 5-HT_{3E} subunits show that all three subunits share key features with other 5-HT₃ subunits (Fig. 4) (Reeves and Lummis, 2002). All of them contain four transmembrane regions with a huge intracellular loop known to affect channel function (Niemeyer and Lummis, 1998). The transmembrane region 2 lines the ion pore which controls ion channel conductance. The ion channel is surrounded by three rings of negatively charged residues and a central ring of small polar residues. They are crucial determinants of ion currents. Unexpectedly, the TM2 region of the 5-HT_{3C}, D as well as E subunits lack a polar residue within the central ring. Furthermore, the 5-HT_{3E} subunit bears a positively charged lysine residue in the anionic cytoplasmic ring. A comparable situation has also been reported in case of the 5-HT_{3B} subunit (Davies et al., 1999). However, the functional consequences have yet to be determined.

Several N-glycosylation and phosphorylation sites are predicted (Fig. 4). N-glycosylation sites have also been reported in the extracellular domain of the known 5-HT_{3A/3B} subunits (Uetz et al., 1994; Isenberg et al., 1993; Miyake et al., 1995; Lankiewicz et al., 1998; Davies et al., 1999) which are involved in receptor assembly (McKernan, 1992; Quirk and Siegel, 2000). Phosphorylation was reported to influence receptor conductance levels and desensitisation rates (van Hooft and Vijverberg, 1995; Hubbard et al., 2000). 5-HT_{3C} and 5-HT_{3E} present a large N-terminal extracellular portion with a cysteine loop with ligand binding capacity. In contrast, the architecture of the putative 5-HT_{3D} subunit is different. It lacks the signal leader sequence and the large N-terminal loop which includes the ligand binding site. This raises the question of whether the 5-HT_{3D} subunit itself is able to form a functional ion channel or is part of a ligand-gated ion channel showing only some of the crucial elements of a 5-HT₃ subunit. Gene identification programs genes and genescan predicted also a virtual *HTR3D* cDNA, which would encode a variant 5-HT_{3D} protein. Since we were not able to verify this hypothetical transcript in more than 20 analysed tissues the expression of the respective transcript remains questionable.

It has been proposed that evolutionary processes influence the dynamics of gene duplication and may in the end lead to genes with newly acquired functions. *HTR3C*, *HTR3D* and *HTR3E* map closely to each other in a region of less than 100 kb suggesting that they have arisen by gene duplication. This is reminiscent of *HTR3A* and *B* which also

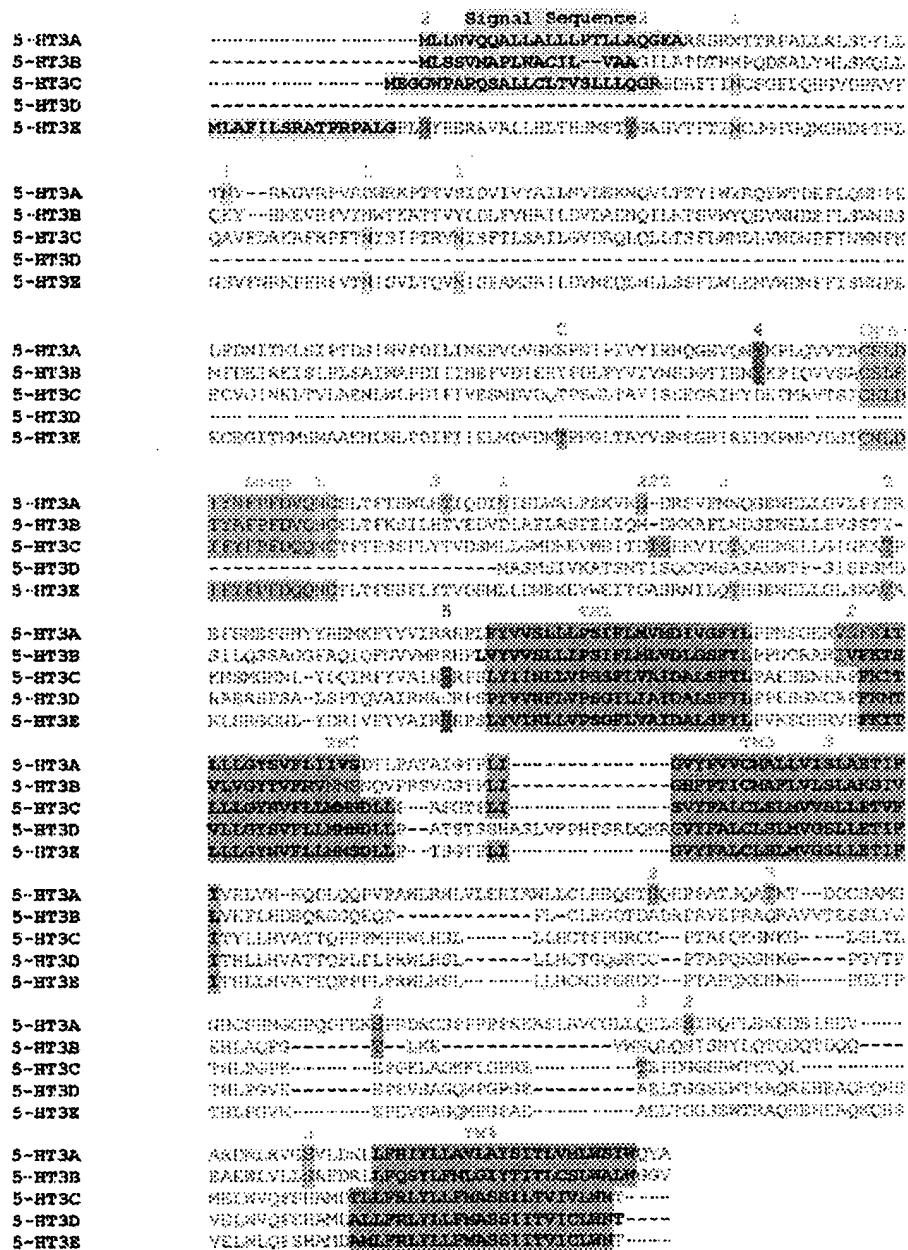


Fig. 4. Multiple sequence alignment of the human 5-HT₃ subunits A, B, C, D and E on protein level. Signal peptide (leader sequence) is marked in light grey, the cysteine loop is indicated in grey and the four transmembrane regions are coloured in dark grey. Putative phosphorylation and glycosylation sites are marked as follows: 1 Asn Glycosylation, 2 Protein Kinase C, 3 Casein Kinase, 4 Tyrosine Kinase, 5 cAMP Kinase.

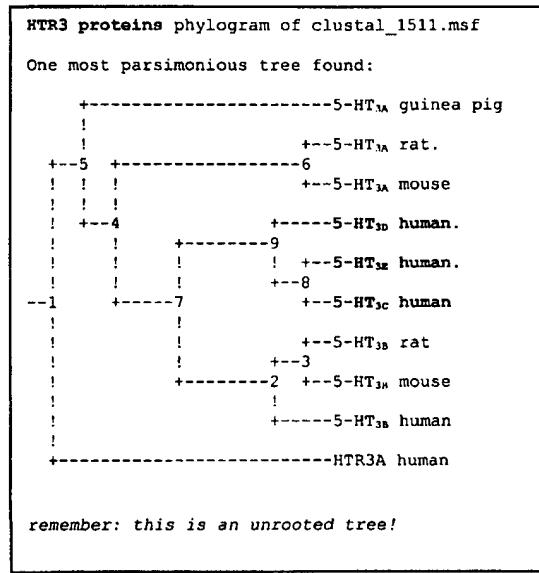
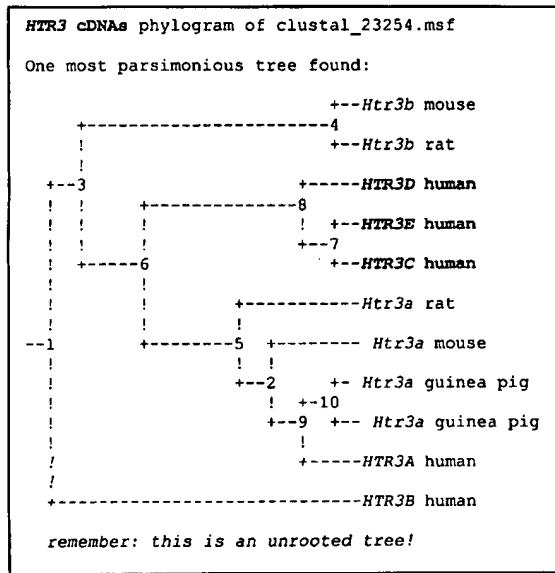
map in close vicinity on chromosome 11q23. *HTR3A* and *B* are structurally very similar with exons almost identical in size and conserved splice sites. Identical exon-intron organisation is shared by *HTR3C* on 3q27, which by sequence comparison is the member most closely related to *HTR3A* and *HTR3B*. Among all the members of the *HTR3* class, *HTR3C*, *HTR3D* and *HTR3E* are the ones most closely

related, suggesting that they diverged later in evolution. This is also predicted by the dendrogram (Table 3) which reveals three major evolutionary branches in this system: one for *HTR3A*, another for *HTR3B* and a third one for *HTR3C*, *HTR3D* and *HTR3E*. It is therefore likely that recent evolutionary processes have shaped these novel genes and that they have acquired novel ultimate functions.

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Table 3
Homologies of *HTR3A*, *B*, *C*, *D* and *E* on nucleotide and protein level

cDNA homology (CDS only)	<i>HTR3A</i> (bestfit/gap; %)	<i>HTR3B</i> (bestfit/gap; %)	<i>HTR3C</i> (bestfit/gap; %)	<i>HTR3D</i> (bestfit/gap; %)
<i>HTR3A</i>	100			
<i>HTR3B</i>	61.2/55.3	100		
<i>HTR3C</i>	60.5/54.4	56.2/47	100	
<i>HTR3D</i>	66.9/47.7	65.7/47.4	84.9/83.3	100
<i>HTR3E</i>	63.4/54.1	55.5/47.8	80.2/82.8	88.6/90.1
Protein homology/identity	5-HT _{3A}	5-HT _{3B}	5-HT _{3C}	5-HT _{3D}
5-HT _{3A}	100			
5-HT _{3B}	56.4/45.9	100		
5-HT _{3C}	48.7/39.8	39.5/30.5	100	
5-HT _{3D}	40.8/35.5	35.0/26.8	69.6/64.8	100
5-HT _{3E}	46.9/39.5	40.8/32.2	79.6/74.3	76.9/73.8



Homologies on nucleotide level were determined by sequence comparison using the respective coding regions. Similarity and identity values were determined using the *bestfit* and *gap* tools of the HUSAR program package from DKFZ, Heidelberg.

To get an idea of the functional role of the different 5-HT₃ receptor subunits, we carried out comparative expression analysis of all *HTR3* genes by RT-PCR. Unlike the known *HTR3* genes which are almost ubiquitously expressed, expression of *HTR3D* is restricted to kidney, colon and liver. *HTR3E* expression was detected in colon and intestine, respectively (Fig. 3). We consider it likely that different subunit compositions of *HTR3C*, *D*, *E* as well as *HTR3A* and *B* in the 5-HT₃ receptor contribute to the complexity of the 5-HT₃ receptor system and therefore explain the varying properties of the respective proteins in different tissues. Whether the diverse properties of the receptor can also be explained by oligomerisation of different receptor subunits in the respective organs remains to be determined. The co-expression of 5-HT_{3A/B} subunits

leading to structurally different 5-HT₃ receptors has recently been shown in neurons of the rat central and peripheral nervous system as well as in mammalian retinae (Morales et al., 2001; Pootanakit and Brunken, 2001; Morales and Wang, 2002). With these new receptors available, expression and functional analysis of recombinant homo- and hetero-oligomeric 5-HT₃ receptor complexes will now provide a new insight into the maturational, structural and functional diversity of the 5-HT₃ receptor system.

Activation of the 5-HT₃ receptors subserves a variety of physiological effects in the central and peripheral neurons (Bloom and Morales, 1998) and it has been predicted that it probably plays a role in a number of human diseases such as anxiety, schizophrenia, depression, migraine, vasospasm, epilepsy, fibromyalgia, bulimia and irritable bowel

syndrome (Graeff, 1997). Since *HTR3D/E* have been shown to be specifically expressed in colon and intestine we hypothesise that these receptor genes are involved in the etiology of diseases of the gastrointestinal tract such as irritable bowel syndrome. Irritable bowel syndrome is associated with abdominal pain and abnormal bowel activity and may reflect hypersensitivity of the gastrointestinal tract to normal stimuli. The basis of the disease is not understood, but there is evidence that serotonin plays a crucial role since it is a major neurotransmitter in the gastrointestinal system. Furthermore, the fact that the 5-HT₃ receptor is involved in the control of gastrointestinal function and that 5-HT₃ receptor antagonists are used in the therapy of irritable bowel syndrome (Humphrey et al., 1999; Jones and Blackburn, 2002) predicts that *HTR3D* and *E* are interesting candidate genes for this disorder.

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